0026-895X/96/051309-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:1309–1315 (1996).

Dopamine Induces Apoptotic Cell Death of a Catecholaminergic Cell Line Derived from the Central Nervous System

JOSEPH M. MASSERANO, LI GONG, HENRIETTA KULAGA, IVORY BAKER, and RICHARD JED WYATT

National Institute of Mental Health Neuroscience Center at Saint Elizabeths, Neuropsychiatry Branch, Washington. D. C. 20032

Received April 18, 1996; Accepted July 31, 1996

SUMMARY

Dopamine produces a time- and dose-dependent increase in cell death in a clonal catecholaminergic cell line (CATH.a) derived from the central nervous system. Cell death also occurred after treatment with the catecholamines L-dihydroxyphenylalanine, norepinephrine, epinephrine, and isoproterenol, as well as the neurotoxic compound 6-hydroxydopamine. Cell death is not receptor mediated because selective noradrenergic and dopaminergic receptor agonists had no effect on CATH.a cell viability. Dopamine induces apoptotic cell death as indicated by DNA fragmentation measured by gel electrophoresis and by flow cytometric analysis. Apoptosis seems to be produced by dopamine autoxidation, because intracellular peroxides increase after dopamine treatment and cell death can be inhibited by catalase and *N*-acetylcysteine. *N*-acetylcysteine produced a dose-dependent decrease in dopamine-induced cell death; this correlated with a decrease in peroxide formation. In addition, antisense to the antioxidant protein bcl-2 increases the sensitivity of CATH.a cells to dopamine-induced cell death. These findings indicate that the oxidative products of dopamine cause neurotoxicity through apoptosis.

Two general mechanisms for cell death are known: necrosis and apoptosis. Necrosis is characterized by mitochondrial swelling and the immediate loss of the plasma membrane integrity leading to cell lysis and an inflammatory response. In contrast, apoptosis is characterized by cell shrinkage, chromatin condensation, systematic DNA cleavage, and phagocytosis of the cell before the integrity of the plasma membrane is lost. Apoptosis is crucial for organogenesis in the developing central nervous system, in which up to 50% of the neurons undergo apoptosis (1). Apoptosis also may occur in the adult rat brain after ischemia (2), seizures (3), adrenalectomy (4), or autoimmune encephalomyelitis (5). It also has been proposed that apoptosis may be a mechanism by which neurons die in some human neurodegenerative diseases (6). The primary cause of one such neurodegenerative disorder, Parkinson's disease, is unknown; however, it is characterized by a selective loss of midbrain dopamine neurons. These neurons are highly sensitive to increases in free radical formation, an event associated with apoptosis (7–9). One source of free radical formation in the Parkinsonian brain may be the autoxidation and metabolism of dopamine (7). Data from rats given the neurotoxins 6-OHDA and methamphetamine suggest that *in vivo* free radical formation within a dopamine neuron may lead to its death (10–12). In primary cultures of catecholamine neurons isolated from the central nervous system dopamine, its precursor L-dihydroxyphenylalanine, norepinephrine, epinephrine, and isoproterenol have been shown to be neurotoxic (13–15). The catecholamine neurons (tyrosine hydroxylase positive) showed greater sensitivity to the neurotoxic effects of catecholamines than other neurons (neuron-specific enolase positive) or glia cells (glial fibrillary acidic protein-positive) in these primary cultures (13). Unfortunately, catecholamine-containing cells comprise only a small percentage of these primary cultures, which makes it difficult to explore the cellular mechanisms for cell death.

To more systematically examine the mechanism of dopamine neurotoxicity, we used a clonal cell line (CATH.a) developed by Suri *et al.* (16). CATH.a cells were derived from a brain tumor of transgenic mice carrying the simian virus 40 T antigen oncogene under the transcriptional control of the rat tyrosine hydroxylase gene. CATH.a cells contain the catecholamine biosynthetic enzymes tyrosine hydroxylase and dopamine-β-hydroxylase in a ratio of 7:1 and produce both dopamine and norepinephrine in a ratio of 5:1. In our laboratory, CATH.a cells accumulate [^{3}H]-dopamine in a temperature-dependent manner (1.3 pmol/min/mg of protein) through a mechanism that is sensitive to the dopamine uptake blockers GBR 12909 (maximal inhibition, 76%) and cocaine (maximal inhibition, 52%). CATH.a cells also stain

ABBREVIATIONS: 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; MAO, monoamine oxidase; DCDHF-DA, 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate, bis(acetoxymethyl) ester.

for the neuronal proteins neurofilament and synaptophysin, suggesting that they contain small storage vesicles, but they do not stain for the astrocyte marker glial fibrillary acidic protein (16).

Materials and Methods

Drugs and chemicals. Dopamine, 6-OHDA, L-dihydroxyphenylalanine, norepinephrine, epinephrine, isoproterenol, methamphetamine, cocaine, pargyline, glutamate, *N*-methyl-D-aspartate, nicotine, dibutyryl cAMP, forskolin, *N*-acetylcysteine, actinomycin D, and cycloheximide were purchased from Sigma Chemical (St. Louis, MO). Quinpirole, phenylephrine, clonidine, albuterol, CGP-12177, SKF 38393, 6-chloro-2-amino-4-phosphonobutanoic acid, SCH 23390, SKF 83566, sulpiride, dantrolene, ω-conotoxin, nifedipine, and BAY K8644 were purchased from Research Biochemicals International (Natick, MA). Catalase was purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals used were American Chemical Society analytical grade.

Tissue culture and treatments. CATH.a cells were obtained from Dr. Chikaraishi (Duke University, Durham, NC). The cells were grown in Roswell Park Memorial Institute 1640 culture medium, supplemented with 4% fetal bovine serum (Hyclone Lab, Logan, UT), 8% horse serum (GIBCO/BRL, Gaithersburg, MD), and 2 mM L-glutamine (GIBCOBRL) in 80-mm^2 culture flasks at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated for experiments on polystyrene tissue culture dishes and allowed to attach for 24 hr. Cells were then treated with fresh media containing several concentrations of compounds for 4–48 hr.

Cell death assay. Cells were plated on polystyrene six-well plates at a density of approximately 600,000–1,000,000 cells per well. After a 24-hr attachment period, fresh media (37°, 5% CO_2) were added with or without treatments. At various times after the treatments, propidium iodide (500 ng, DNA QC Kit; Becton Dickinson, San Jose, CA) was added to the media (final concentration, 1 μM) and the cells were kept for 40 min in a CO_2 incubator (37°, 5% CO_2). Cells in the media and on the plates were removed, pelleted, and resuspended in 0.5 ml of Dulbecco's PBS without calcium chloride and magnesium chloride (Sigma), kept at 4°, protected from light, and assayed within 1 hr. From each sample, 10,000 cells were analyzed with a FACScan flow cytometer (Becton Dickinson) with the FL-3 detector in log. Data were analyzed with LYSYS II software (Hewlett Packard, Palo Alto, CA). Cell death is presented as the percentage of cells that took up propidium iodide.

Gel analysis of DNA fragmentation. CATH.a cells were untreated or treated with 500 μM dopamine for 24 hr. Ten million to 20 million cells were pelleted and resuspended in lysis buffer (10 mM Tris·HCl, pH 7.5, 40 mM EDTA, 0.5% Triton X-100) and incubated at room temperature for 15 min, at 50° for 60 min with 50 μg/ml RNase A (Boehringer Mannheim), and at 50° for 60 min with 0.5 mg/ml of proteinase K (Oncor, Gaithersburg, MD). The digested samples were extracted once with an equal volume of saturated phenol and twice with chloroform, and DNA in the supernatant was precipitated at −20° overnight after the addition of 3.0 M sodium acetate/75% ethanol. DNA was pelleted by microcentrifugation, dried, and resuspended in distilled water. Aliquots (12 μg) of DNA were electrophoresed in a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide in Tris/borate/EDTA buffer (89 mM Tris/borate, 2 mM EDTA, pH 8.3). DNA fragments were visualized by UV transillumination. DNA molecular weight reference preparations (123 base-pair stepladder; Sigma) were run in parallel to estimate the size of the DNA fragments.

FACScan analysis of DNA fragmentation. Cells were untreated or treated with dopamine (500 μM) for 24 hr. The cells were permeabilized and DNA was subjected to an *in situ* tailing reaction in which residues of digoxigenin nucleotides were added catalytically to the DNA by terminal deoxynucleotidyl transferase. Fluoresceinated antidigoxigenin antibody was used to detect the labeled DNA. These procedures were performed using the ApopTag *In Situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD). Ten thousand cells were analyzed using a FACScan flow cytometer and the data were analyzed with LYSYS II software. Gates were set to quantify the number of cells undergoing DNA fragmentation as described in Fig. 4.

Assay of intracellular peroxides. Cells (1–5 × 10^6) were untreated or treated with dopamine (500 μM) and were loaded with 5 μM DCDHF-DA (Molecular Probes, Eugene, OR) for 1 hr in a CO_2 incubator (37°, 5% CO_2) before each assay. In some experiments, 10 μg/ml catalase was added 2 hr before the addition of dopamine. Cells in the media and on the plates were pelleted, washed with PBS, and resuspended in PBS for analysis. Ten thousand cells per sample were analyzed using a FACScan flow cytometer and data were analyzed using LYSYS II software.

bcl-2 antisense experiments. bcl-2 antisense (5′-TCT-CCC-GGC-TTG-CGC-CAT-3′) and sense (5′-ATG-GCG-CAA-GCC-GGG-AGA-3′) phosphorothioate oligodeoxynucleotides were made by Quality Controlled Biochemicals (Hopkinton, MA). Oligonucleotides were mixed with the transfection reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N, N, N,*-trimethylammonium methylsulfate (Boehringer Mannheim) according to the manufacturer's directions immediately before addition to the cell cultures. CATH.a cells were plated at a density of 0.5 × 10^{-6} cells per 35-mm dish (day 0), and 24 hr later, the media were changed and 10 μM antisense or sense oligonucleotides were added on days 1, 2, and 3. Twenty-four hours after the last addition on day 4, the media were changed and 3 hr later dopamine was added. Approximately 20 hr later, cell death was determined.

Results

Catecholamines produce cell death in a catecholaminergic cell line. Incubation of the CATH.a cells with dopamine (250 μM) produced a time-dependent cell death with 20% and 75% cell death at 4 and 48 hr, respectively. Concentrations of dopamine from 50 μM to 500 μM produced a dose-dependent cell death in the CATH.a cells (Fig. 1). 6-OHDA (100 μM) produced cell death equivalent to the highest doses of dopamine (250 μM and 500 μM). Cell death was not selective for dopamine because the catecholamines L-dihydroxyphenylalanine, norepinephrine, epinephrine, and isoproterenol produced similar cell death in the CATH.a cells (Fig. 2). The possibility that catechol-induced cell death of CATH.a cells was receptor mediated was evaluated by testing a number of catecholamine receptor agonists (Table 1). The selective catecholamine receptor agonists for dopamine D_1 and D_2 receptors and α_1-adrenergic

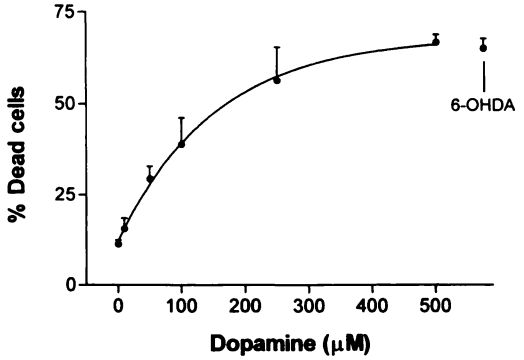

Fig. 1. Effects of dopamine on cell death in CATH.a cells. Cells were untreated or treated with dopamine (10–500 μM) or 6-OHDA (100 μM) for 24 hr. Percentage of dead cells was determined by flow cytometric analysis of propidium iodine uptake as described in Materials and Methods. The results are mean ± standard error from 5 to 15 experiments.

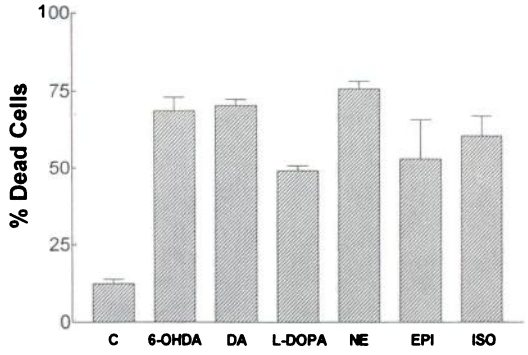

Fig. 2. Effects of catecholamines on cell death in clonal catecholaminergic (CATH.a) cells. Cells were untreated (*C*, control) or treated with 500 μM 6-OHDA, dopamine (*DA*), L-dihydroxyphenylalanine (*L-DOPA*), norepinephrine (*NE*), epinephrine (*EPI*), and isoproterenol (*ISO*) for 24 hr. Percentage of dead cells was determined by flow cytometric analysis of propidium iodine uptake as described in Materials and Methods. The results are means ± standard error from four to eight experiments.

TABLE 1

Effects of noncatechol compounds on cell death in CATH.a cells

Cells were treated with the various compounds for 24 hr and cell death was determined as described in Materials and Methods. Concentrations were 500 μM unless otherwise listed.

Treatment	Percentage of Cell Death
Control	9 ± 2
SKF 38393 (DA_1 agonist) (25 μM)	15 ± 3
6-Chloro-2-amino-4-phosphobutanoic acid (DA_1 agonist) (25 μM)	12 ± 3
Quinpirole (DA_2 agonist)	13 ± 3
Phenylephrine (α_1 agonist)	8 ± 2
Clonidine (α_2 agonist)	10 ± 2
Albuterol (β_1 agonist)	23 ± 1[a]
CGP-12177 (β_2 agonist)	22 ± 3[a]
Methamphetamine	23 ± 4[a]
Cocaine	36 ± 4[a]
Glutamate (1 mM)	12 ± 6
N-methyl-D-aspartate	8 ± 2
BAY K 8644 (Ca^{2+} ↑, 10 μM)	36 ± 4[a]
Nicotine	7 ± 2
H_2O_2	76 ± 3[a]
Serum-free media	73 ± 3[a]

[a] Significantly different from control, $p < 0.05$ using the Student *t* test.

and α_2-adrenergic receptors had no effect on cell death. The β_1-adrenergic and β_2-adrenergic receptor agonists produced a slight increase in cell death. A number of other compounds were also tested that have been shown to be neurotoxic to various neuronal cell types *in vitro* and/or *in vivo* (Table 1). Exposure of the CATH.a cells to H_2O_2 or serum-free media produced cell death comparable with the catecholamines. Two drugs that are often abused, cocaine and methamphetamine, which affect the release and/or uptake of catecholamines, also produced a moderate increase in cell death. Receptor-mediated drugs that increase sodium and calcium in neurons had no effects on cell death (nicotine, glutamate, and *N*-methyl-D-aspartate), whereas BAY K 8644, a non–receptor-mediated calcium channel agonist, produced 36% cell death. A number of compounds were evaluated for their ability to prevent dopamine-induced cell death (Table 2). Compounds that had no effect on dopamine-induced cell death include pargyline, an MAO inhibitor; SCH 23390, SCH 83566, and sulpiride, dopamine receptor antagonists; nifedipine, ω-conotoxin, and dantrolene, compounds that decrease

TABLE 2

Compounds that did not inhibit dopamine-induced cell death in CATH.a cells

Compounds were added to the media 2 hr before the addition of dopamine (250 μM). Cell death was determined 24 hr later as described in Materials and Methods.

Compound	Dose	Mechanism
Pargyline	100 μM	MAO ↓
SCH 23390	10 μM	D_1 receptor ↓
SCH 83566	10 μM	D_1 receptor ↓
Sulpiride	10 μM	D_2 receptor ↓
Nifedipine	10 μM	L-type Ca^{2+} channel ↓
ω-conotoxin	0.5 μM	N-type Ca^{2+} channel ↓
Dantrolene	50 μM	Ca^{2+} intracellular ↓
Actinomycin D	2 μM	RNA synthesis ↓
Cycloheximide	10 μg/ml	Protein synthesis ↓
Forskolin	20 μM	cAMP ↑
D-cAMP	2 mM	cAMP ↑

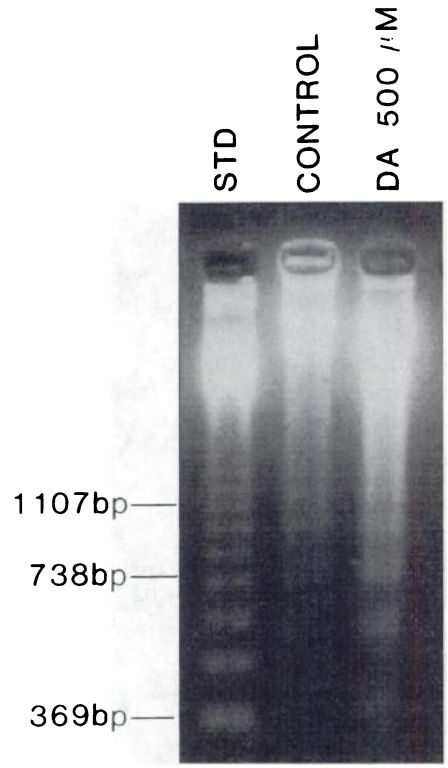

Fig. 3. Effects of dopamine on DNA fragmentation. CATH.a cells were untreated or treated with 500 μM dopamine for 24 hr. DNA was isolated from the cells and 12 μg of protein was run on a 1.5% agarose gel as described in Materials and Methods. The standard lane consists of a 123 base-pair DNA stepladder. Data shown are from a representative study repeated five times with comparable results.

intracellular calcium; actinomycin D, an inhibitor of RNA synthesis; cycloheximide, an inhibitor of protein synthesis; forskolin and dibutyryl cAMP, compounds that enhance cAMP actions in cells and have been shown to protect cultures of sympathetic neurons, hypothalamic neurons, and cerebellar granule neurons from cell death (17–19).

Dopamine produces apoptosis in CATH.a cells. After incubation of CATH.a cells with 500 μM dopamine for 24 hr, gel analysis showed DNA fragmentation that was indicative of apoptosis (Fig. 3). Fig. 4 (*right*) shows DNA staining with propidium iodide in untreated and dopamine-treated cells. The G_0/G_1 and G_2/M peaks represent intact DNA in the cell

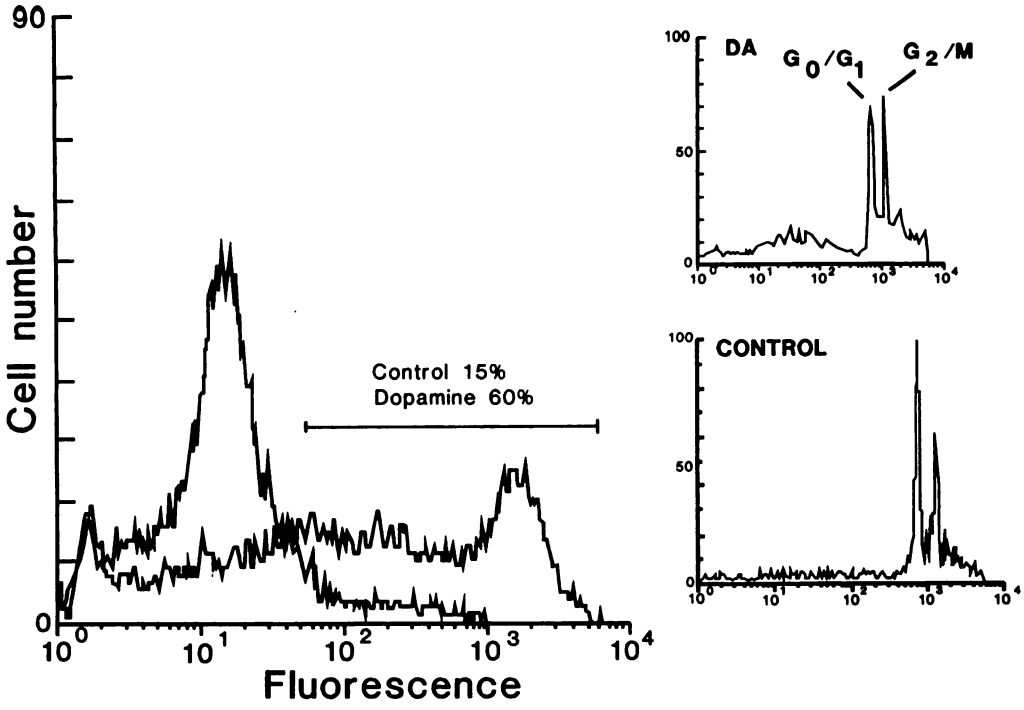

Fig. 4. FACScan analysis of DNA fragmentation. Cells were untreated or treated with dopamine (*DA*, 500 μM) for 24 hr. *Right*, DNA histograms obtained from cultures of CATH.a cells with or without dopamine. Fragmented DNA can be seen to the *left* of the G_0/G_1 cell cycle peak. *Left*, representative example of a flow cytometric analysis of apoptosis as detected by DNA strand breaks. Fluoresceinated antidigoxigenin antibody was used to detect the labeled DNA using the Apotag *In Situ* Apoptosis Detection Kit. Ten thousand cells were evaluated from each sample. Percentage of cells containing apoptotic DNA was calculated by setting the gate at the overlap of the control and dopamine-treated samples. The control is the high peak to the *left* and the dopamine-treated sample is the relative high peak to the *right*. *Horizontal bar*, 60% of the dopamine-treated cells and 15% of the control cells, which showed labeling with fluoresceinated antidigoxigenin antibody.

cycle and the lower molecular weight fragments to the left of the G_0/G_1 peak represent potential apoptotic DNA. Clarification and quantification of this apoptotic DNA were determined by flow cytometric analysis of DNA strand breaks measured after an *in situ* tailing reaction. A measurement of apoptotic cells using this technique is shown in Fig. 4 (*left*). In this sample, approximately 15% of the control cells and 60% of the dopamine-treated cells show labeling of the fragmented DNA. Using this analysis, there was a dose-dependent increase in apoptotic cells after treatment with dopamine (Fig. 5).

Dopamine increases peroxide formation. The possibility that cell death in the CATH.a cells was produced by free radical formation was evaluated by measuring intracellular peroxide formation. Fig. 6 illustrates a dose-dependent increase in peroxide formation in the CATH.a cells with increasing concentrations of dopamine (50–500 μM). 6-OHDA (100 μM) produced changes similar to 250 μM and 500 μM dopamine. The addition of catalase (10 μg/ml of media), an

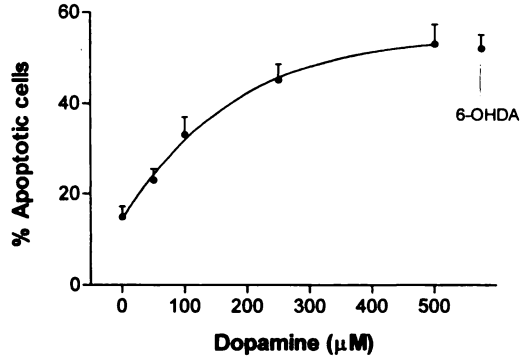

Fig. 5. The percentage of CATH.a cells showing apoptosis. Cells were untreated or treated with dopamine (10–500 μM) or 6-OHDA (100 μM) for 24 hr. Calculations of apoptotic cells were made using flow cytometric analysis as described in Materials and Methods and illustrated in Fig. 4. The results are means ± standard error from four experiments.

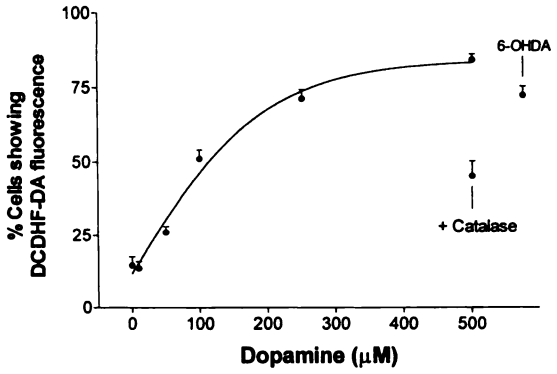

Fig. 6. Effects of dopamine on peroxide formation in CATH.a cells. Cells were untreated or treated with dopamine (10–500 μM) or 6-OHDA (100 μM) for 24 hr. Catalase (10 μg/ml) was added 2 hr before dopamine (500 μM). DCDHF-DA was added to the media 1 hr before processing of the cells for flow cytometric analysis. The results are means ± standard error of three experiments.

enzyme that promotes the breakdown of H_2O_2 to H_2O and O_2, inhibited dopamine-induced peroxide formation (Fig. 6) and cell death (Fig. 7). The addition of the antioxidant *N*-acetylcysteine (1 mM) to the media 2 hr before dopamine also inhibited dopamine-induced cell death in the CATH.a cells. *N*-acetylcysteine (100–1250 μM) produced a dose-dependent protection from dopamine-induced cell death in the CATH.a cells; this correlated with a decrease in dopamine-induced peroxide formation (Fig. 8). IC_{50} values for *N*-acetylcysteine were 276 μM for inhibition of peroxide formation and 367 μM for inhibition of cell death. *N*-acetylcysteine alone in concentrations up to 1250 μM had no effect on cell viability (percentage of dead cells, 10.9 ± 0.6) and no effect on peroxide formation (percentage of cells showing DCDHF-DA fluorescence, 9.8 ± 0.8).

Antisense oligonucleotide to bcl-2 increases the sensitivity of CATH.a cells to dopamine. bcl-2 protein has been shown to have a protective effect on cell death produced

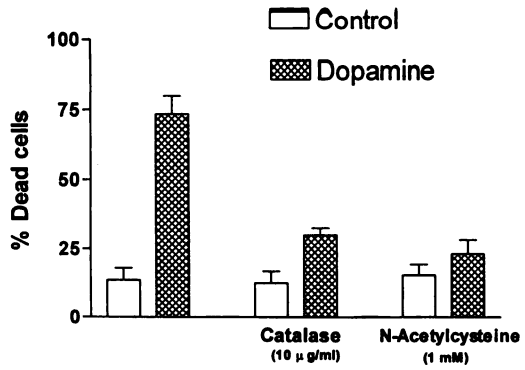

Fig. 7. Effects of catalase and *N*-acetylcysteine on dopamine-induced cell death in CATH.a cells. Cells were treated with catalase (10 μg/ml media) or *N*-acetylcysteine (1 mM) and 2 hr later, dopamine (*DA*, 500 μM) was added. Twenty-four hours later, the percentage of dead cells was determined using flow cytometric analysis as described in Materials and Methods. The results are means ± standard error of six to eight experiments.

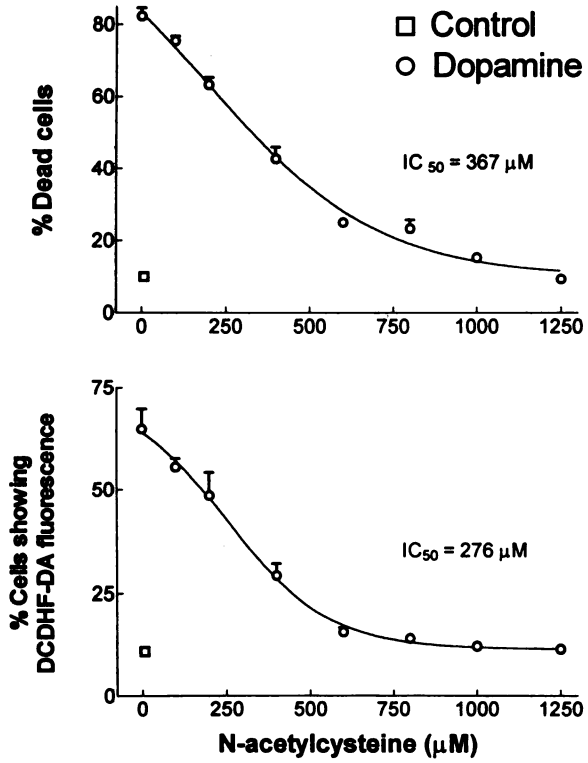

Fig. 8. Effects of *N*-acetylcysteine on dopamine-induced cell death and peroxide formation. *N*-acetylcysteine (100–1250 μM) was added to the media 2 hr before the addition of dopamine (250 μM). Twenty-four hours later, the cells were processed for analysis of cell death or peroxide formation as described in Materials and Methods. *N*-acetylcysteine alone in concentrations up to 1250 μM had no effect on cell viability or peroxide formation. The results are means ± standard error of four to six experiments.

by H_2O_2 in a number of neuronal systems (20, 21). Western analysis indicated that CATH.a cells contain basal levels of the bcl-2 protein. Incubation of CATH.a cells with antisense to bcl-2 for 3 days enhanced base-line cell death and dopamine (50–250 μM) induced cell death (Fig. 9). At the time of assay, antisense to bcl-2 lowered bcl-2 levels by 40% as measured by Western analysis. Incubation with the sense oligonucleotide to bcl-2 had no effect on dopamine-induced cell death (data not shown).

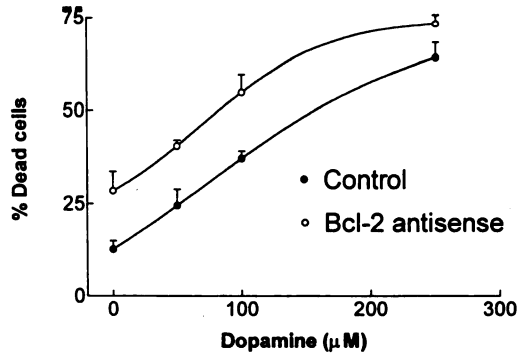

Fig. 9. Effects of bcl-2 antisense oligonucleotides on dopamine-induced cell death in CATH.a cells. Cells were untreated or treated with Bcl-2 antisense oligonucleotide every 24 hr for 3 days. Media was changed and dopamine was added. Twenty hours later, the percentage of dead cells was determined using flow cytometric analysis as described in Materials and Methods. The results are means ± standard error of three experiments.

Discussion

Dopamine produces cell death in the CATH.a cells at concentrations ranging from 50 to 500 μM. Whether this occurs in the intact animal is critical. Catecholamine content in dopamine cell bodies is estimated to be approximately 0.1–1.0 mM (22); however, most of these catecholamines are protected by sequestration in vesicles (23). The quantity of catecholamines in the synapse necessary to produce a physiological response [low micromolar (24)] is unlikely to cause neurotoxicity. Nevertheless, certain pathological conditions, such as ischemia and hypoxia, have been shown to increase dopamine release (700–1100%) in the striatum of animals, which leads to synaptic concentrations of catecholamines that could be potentially damaging to the brain (25, 26). A number of studies suggest that autoxidation of catecholamines does occur in the brain (27). The most apparent evidence for this autoxidation is that melanin, a byproduct of autoxidized catecholamines, accumulates with age in a number of catecholamine cell-body regions, including the substantia nigra, ventral tegmental area, and locus ceruleus. Dopamine can be metabolized by either autoxidation to the dopamine orthoquinone-generating H_2O_2, O_2^-, and OH· or through enzymatic metabolism by MAO, which also can generate H_2O_2. Autoxidation of dopamine seems to be responsible for the neurotoxicity in the CATH.a cells because the catecholamine isoproterenol, which is not a substrate for MAO, produces similar toxicity in these cells (62% death at 500 μM) (Fig. 2) and pargyline (100 μM), an inhibitor of MAO, had no effect on dopamine-induced cell death. The direct addition of H_2O_2 to the culture media also produced cell death and apoptosis in the CATH.a cells (Table 1). Similar apoptotic cell death induced by H_2O_2 has been shown to occur in a number of tumor cell lines (28) and primary cultures of rat cortex (29). In the CATH.a cells, dopamine increased intracellular peroxides and cell death was inhibited by the addition of catalase or *N*-acetylcysteine to the media. Catalase and *N*-acetylcysteine also decreased intracellular peroxide formation produced by dopamine. Other possible explanations for cell death, such as an increase in intracellular calcium or synthesis of a "cell death" protein, were excluded because drugs that affect cellular calcium levels [nifedipine (10 μM), ω-conotoxin (0.5 μM), dantrolene (50 μM)], inhibition

of RNA synthesis by actinomycin D (2 μg/ml), or protein synthesis by cycloheximide (10 μg/ml) had no effect on dopamine-induced cell death. In addition, dopamine-induced cell death was not receptor-mediated because it could not be prevented by prior incubation of the cells with D_1 dopamine receptor (SCH23390, SKF83566) or D_2 dopamine receptor (sulpiride) antagonists. Therefore, we conclude that dopamine produces apoptotic cell death in the CATH.a catecholamine neurons by the formation of intracellular peroxides. The brain contains enzymes that protect catecholamine neurons from damage produced by autoxidation, including superoxide dismutase, glutathione peroxidase, and catalase. In disease states, however, it can be hypothesized that abnormal activity of these protective enzymes may lead to enhanced oxidative stress on the neurons. This may be the case in Parkinson's disease, in which postmortem studies indicate that catalase and glutathione peroxidase, as well as levels of the substrate glutathione, are decreased in the substantia nigra, which suggests that the substantia nigra neurons of such individuals may be more susceptible to oxidative stress (30, 31). The possibility that oxidative stress in the Parkinsonian brain could lead to the observed cell death through an apoptotic mechanism has not been evaluated; however, apoptotic cells have been identified in postmortem hippocampus obtained from patients with Alzheimer's disease, which suggests that this may be a mechanism for neurodegenerative cell death (6).

Recently, the bcl-2 protein has been found in neurons in the adult brain and has been hypothesized to play a role as an antidote to programmed death in these long-lived cells (32, 33). bcl-2 is believed to function in an antioxidant pathway to prevent apoptosis (20, 21). Several studies find that cells that do not normally express bcl-2 can be protected from apoptosis when the bcl-2 protein is overexpressed in the cells in culture (20, 34, 35). bcl-2 is also protective *in vivo* against cell death produced by experimental ischemia and motoneuron axotomy in transgenic mice overexpressing the protein (36, 37). In addition, inhibition of bcl-2 synthesis using antisense oligonucleotides results in an increase in apoptosis and/or an increase susceptibility of cells to stimuli that produce cell death (38, 39). Similarly, the present study found a potentiation of basal and dopamine-induced cell death in the CATH.a cells after treatment with antisense to bcl-2. Efforts to define regulators of bcl-2 in the CATH.a cells may provide information on deterring neurodegenerative cell loss.

The interrelationships among our findings of dopamine-induced apoptosis, the increase in peroxide formation, and bcl-2 levels require further investigation to ascertain more definitively the functional significance of these changes in neurodegenerative diseases.

Acknowledgments

We thank Dona Chikaraishi for supplying us with the CATH.a cells and for valuable discussions and technical advice.

References

1. Raff, M. C., B. A. Barres, J. F. Burne, H. S. Coles, Y. Ishizaki, and M. D. Jacobson. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science (Washington D. C.)* **262:**695–700 (1993).
2. MacManus, J. P., I. E. Hill, Z. G. Huang, I. Rasquinha, and E. Preston. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci. Lett.* **164:**89–92 (1993).
3. Pollard, H., S. Cantagrel, C. Charriaut-Marlangue, J. Moreau, and Y. Ben Ari. Apoptosis associated DNA fragmentation in epileptic brain damage. *Neuroreport* **5:**1053–1055 (1994).
4. Sloviter, R. S., E. Dean, and S. Neubort. Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. *J. Comp. Neurol.* **330:**337–351 (1993).
5. Gold, R., M. Schmied, G. Giegerich, and H. Breitschopf. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab. Invest.* **71:**219–225 (1994).
6. Su, J. H., A. J. Anderson, B. J. Cummings, and C. W. Cotman. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* **5:**2529–2533 (1994).
7. Olanow, C. W. A radical hypothesis for neurodegeneration. *Trends Neurosci.* **16:**439–444 (1993).
8. Adams, J. D., and I. N. Odunze. Oxygen free radicals and Parkinson's disease. *Free Radical Biol. Med.* **10:**161–169 (1991).
9. Hirsch, E. C. Does oxidative stress participate in nerve cell death in Parkinson's disease? *Eur. Neurol.* **33**(Suppl. 1):52–59 (1993).
10. De Vito, M. J., and G. C. Wagner. Methamphetamine-induced neuronal damage a possible role for free radicals. *Neuropharmacol.* **28:**1145–1150 (1989).
11. Cohen, G., and R. E. Heikkila. The generation of hydrogen peroxide, superoxide radical and hydroxy radical by 6-hydroxydopamine, dialuric acid and related cytotoxic agents. *J. Biol. Chem.* **249:**2447–2452 (1974).
12. Gibb, J. W., M. Johnson, D. M. Stone, and G. R. Hanson. Mechanisms mediating biogenic amine deficits induced by amphetamine and its congeners. *Nat. Inst. Drug Abuse Res. Monogr.* **136:**226 (1993).
13. Louis, J.-C., E. Magal, and S. Varon. Receptor-mediated toxicity of norepinephrine on cultured catecholaminergic neurons of the rat brain stem. *J. Pharmacol. Exp. Ther.* **262:**1274–1283 (1992).
14. Michel, P. P., and F. Hefti. Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J. Neurosci. Res.* **26:**428–435 (1990).
15. Mytilineou, C., S.-K. Han, and G. Cohen. Toxic and protective effects of L-DOPA on mesencephalic cell cultures. *J. Neurochem.* **61:**1470–1478 (1993).
16. Suri, C., B. P. Fung, A. S. Tischler, and D. M. Chikaraishi. Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. *J. Neurosci.* **13:**1280–1291 (1993).
17. Deckwerth, T. L., and E. M. Johnson, Jr. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell. Biol.* **123:**1207–1222 (1993).
18. De, A., N. I. Boyadjieva, M. Pastorcic, B. V. Reddy, and D. K. Sarkar. Cyclic AMP and ethanol interact to control apoptosis and differentiation in hypothalamic beta-endorphin neurons. *J. Biol. Chem.* **269:**26697–26705 (1994).
19. D'Mello, S. R., C. Galli, T. Ciotti, and P. Caslissano. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc. Natl. Acad. Sci. USA* **90:** 10989–10993 (1993).
20. Hockenbery, D. M., A. N. Oltvai, X-M Yin, C. L. Milliman, and S. J. Korsmeyer. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75:**241–251 (1993).
21. Kane, D. J., T. A. Sarafian, R. Anton, H. Hahn, E. B. Gralla, J. S. Valentine, T. Ord, and D. E. Bredesen. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Washington D. C.)* **262:**1274–1277 (1993).
22. Jonsson, G. Quantitation of fluorescence of biogenic monoamines. *Prog. Histochem. Cytochem.* **2:**299–344 (1971).
23. Beaudet, A., and L. Descarries. Fine structure of monoamine axon terminals in cerebral cortex, in *Monoamine Innervation of Cerebral Cortex* (L. Descarries, T. R. Reader, and H. H. Jasper, eds.). Liss, New York, 109–127 (1984).
24. Madison, D. V., and R. A. Nicoll. Noradrenalin blocks accommodation of pyramidal cell discharge in the hippocampus. *Nature (Lond.)* **299:**636–638 (1982).
25. Baker, A. J., M. H. Zornow, M. S. Scheller, T. L. Yaksh, S. R. Skilling, D. H. Smulli, A. A. Larson, and R. Kuczenski. Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin and dopamine metabolites after transient global ischemia in the rabbit brain. *J. Neurochem.* **57:**1370–1379 (1991).
26. Akiyama, Y., K. Koshimura, T. Ohue, K. Lee, S. Miwa, S. Yamagata, and H. Kikuchi. Effects of hypoxia on the activity of the dopaminergic neuron system in the rat striatum as studied by in vivo brain microdialysis. *J. Neurochem.* **57:**997–1002 (1991).
27. Graham, D. G. Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. *Neurotoxicology* **5:**83–96 (1984).
28. Lennon, S. V., S. J. Martin, and T. G. Cotter. Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. *Cell Prolif.* **24:**203–214 (1991).
29. Whittemore, E. R., D. T. Loo, and C. W. Cotman. Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. *Neuroreport* **5:**1485–1488 (1994).
30. Ambani, L. M., M. H. Van Woert, and S. Murphy. Brain peroxidase and catalase in Parkinson disease. *Arch. Neurol.* **32:**114–118 (1975).

31. Perry, T. L., D. V. Godin, and S. Hansen. Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* **33**:305–310 (1982).
32. Hockenbery, D. M., M. Zutter, W. Hickey, M. Nahm, and S. J. Korsmeyer. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc. Natl. Acad. Sci. USA* **88**:6961–6965 (1991).
33. Abe-Dkohmae, S., N. Harada, D. Yamada, and R. Tanaka. Bcl-2 gene is highly expressed during neurogenesis in the central nervous system. *Biochem. Biophys. Res. Commun.* **191**:915–921 (1993).
34. Mah, S. P., T. Zhong, Y. Liu, A. Roghani, R. H. Edwards, and D. E. Bredesen. The protooncogene bcl-2 inhibits apoptosis in PC12 cells. *J. Neurochem.* **60**:1183–1186 (1993).
35. Allsopp, T. E., S. Wyatt, H. F. Paterson, and A. M. Davies. The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* **73**:295–307 (1993).
36. Martinou, J. C., M. Dubois-Dauphin, J. K. Staple, I. Rodriquez, H. Frankowski, M. Missotten, P. Alabertini, D. Talabot, S. Catsicas, C. Pietra, and J. Huarte. Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* **13**:1017–1030 (1994).
37. Dubois-Dauphin, M., H. Frankowski, Y. Tsujimoto, J. Huarte, and J. C. Martinou. Neonatal motor neurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. *Proc. Natl. Acad. Sci. USA* **91**:3309–3313 (1994).
38. Keith, F. J., D. A. Bradbury, Y.-M. Zhu, and N. H. Russell. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases the sensitivity of AML blasts to Ara-C. *Leukemia* **9**:131–138 (1995).
39. Kitada, S., T. Miyashita, S. Tanaka, and J. C. Reed. Investigations of antisense oligonucleotides targeted against bcl-2 RNAs. *Antisense Res. Dev.* **3**:157–169 (1993).

Send reprint requests to: Joseph M. Masserano, Ph.D., NIMH Neuroscience Center at St. Elizabeths, Neuropsychiatry Branch, 2700 Martin Luther King Jr. Avenue, Washington, DC 20032. E-mail: masseraj@dirpc.nimh.nih.gov